REMARKS

Claims 32-36, 38-41, 52 and 53 are pending. Claims 42-44 have been cancelled by the instant amendment. Claims 1-31, 37, and 45-51 were earlier cancelled. Applicants have amended claim 32. Support for the amendments can be found throughout the specification and in the claims as originally filed. No new matter is introduced by these amendments.

Claims Rejections – 35 U.S.C. §112, first paragraph

It is alleged that claims 42-44 fail to comply with the written description requirement. It is suggested that the claims contain subject matter not adequately described in the specification. Applicants traverse and submit that the claims fully satisfy the requirements of 35 U.S.C. §112, second paragraph. However, in order to expedite allowance of the application, claims 42-44 have been cancelled. The rejection is therefore rendered moot.

Claims Rejections – 35 U.S.C. §112, second paragraph

It is alleged that claims 32-36, 38-44, 52, and 54 fail to particularly point out and distinctly claim the subject matter of the instant invention. Specifically, the Office Action alleges that the term "metabolites" renders claim 32 indefinite. Applicants traverse, but in order to expedite allowance of the application, claim 32 has been amended to remove recitation of "metabolites." The rejection is thus overcome.

Rejection under 35 U.S.C. § 103(a)

Claims 32-36, 38-44, 52, and 53 are rejected as obvious over Sakanaka et al. (Jpn. J. Pharmacol. 67, Suppl. I, 297P, 1995), in view of Liu et al. (US Patent 4,708,949), and in view of

Zhang et al. (Acta Pharmacologica Sinica 17(1), 44-48, 1996). Applicants disagree and respectfully traverse.

It is asserted in the Action that: (1) Sakanaka teaches the use of ginseng saponins and ginsenoside Rb1 to prevent ischemia-induced learning disability and rescued ischemic hippocampus CA1 neuron in gerbils; (2) Liu teaches methods of treating a patient suffering from cerebrovascular disease and impaired neurofunction with a pharmaceutical composition comprising ginsenoside; and (3) Zhang teaches that ginsenoside Rb1 protected rat brains from cerebral infarction. It is then concluded that the combination of (1), (2), and (3) renders the Applicants' instantly claimed subject matter *prima facie* obvious, in part because (1) also provides the suggestion that ginsenoside Rb1 is a neuroprotective molecule.

To establish a *prima facie* case of obviousness, three criteria need be met: (i) there must be a suggestion or motivation to modify the reference or combine the teachings; (ii) there must be a reasonable expectation of success; and (iii) the prior art reference must teach or suggest all the claim limitations. See, MPEP 2143. Applicants submit that the required criteria are not met in the present rejection.

Applicants wish to point out that the Sakanaka reference is an abstract of a presentation, and the full contents of the presentation were disclosed in Wen, T-C. et al. Acta Neuropathol (1996), 91, p. 15-22. A copy of the Wen reference is provided with this response.

Regarding the allegation that the only difference between the Sakanaka reference and the instant invention is a teaching of the effective concentration of ginsenoside Rb1, Applicants wish to point out that neither the Sakanaka reference nor the Wen reference teach or suggest a method of treating a traumatic or compression injury of a nervous tissue, and therefore are distinct from the instant invention. In fact, the Sakanaka and Wen references indicate that attempts to treat transient

forebrain ischemia with ginsenoside Rb1 were ineffective (Wen et al. page 19, left column, lines 3-4). Both the Sakanaka and Wen references merely teach that ginsenoside Rb1 can be used to prevent ischemic nerve cell death. The transient forebrain ischemia model is considered to be less severe than the traumatic or compression injury of a nervous tissue of the instant invention. Traumatic injuries treated by the methods of the invention, such as spinal cord injuries, are considered much more severe in scope than examples of transient forebrain ischemia, such as temporary cerebral blood circulation. There is no teaching or suggestion provided by the Sakanaka reference or the Wen reference that ginsenoside Rb1 can be used to treat a traumatic or compression injury of a nervous tissue.

It is also alleged that the Liu reference teaches cerebral vascular disease using a pharmaceutical composition comprising ginsenoside. As the reference is understood, a four-component composition is administered to treat a cerebral vascular disease, wherein ginsenoside extract is one component. There is no teaching or suggestion that the ginsenoside component is the active compound in any treatment of cerebral vascular disease. Additionally, there is no teaching or suggestion that the composition using ginsenoside Rb1 is effective to treat cerebral vascular disease, wherein the ginsenoside Rb1 is the pharmaceutically active species. Therefore, any combination of the Sakanaka reference with Liu does not provide a motivation or reasonable expectation of success to arrive at the method described in Applicant's amended claim 32. Reconsideration and withdrawal of the rejection is respectfully requested.

It is further alleged that Zhang teaches specific concentrations of ginsenoside Rb1, when used in rats to prevent cerebral infarction. Zhang, in combination with the Sakanaka reference, does not teach or suggest that ginsenoside Rb1 can be used to treat a traumatic or compression injury of a nervous tissue. No motivation is provided by the combination of the Sakanaka reference and Zhang to treat traumatic or compression injury of a nervous tissue, as both references simply provide for methods of prevention. Additionally, no reasonable expectation of

success is provided by the combination of the Sakanaka reference and Zhang as both references only provide for methods of preventing ischemic nerve cell death or cerebral infarction. Reconsideration and withdrawal of the rejection is respectfully requested.

In view of the above remarks, Applicants believe the pending application is in condition for allowance. Should any of the claims not be found to be allowable, the Examiner is requested to telephone Applicants' undersigned representative at the number below. Applicants thank the Examiner in advance for this courtesy.

The Director is hereby authorized to charge or credit any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 04-1105, under Order No. 71526-56238.

Respectfully submitted,

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REGULAR PAPER

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Ginseng root prevents learning disability and neuronal loss in gerbils with 5-minute forebrain ischemia

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Abstract The present study was designed to investigate the possible neuroprotective activity of ginseng roots in 5-min ischemic gerbils using a step-down passive avoidance task and subsequent neuron and synapse counts in the hippocampal CA1 region. The following drugs were administered for 7 days before the induced ischemia: red ginseng powder (RGP), crude ginseng saponin (CGS), crude ginseng non-saponin (CGNS), and pure ginsenosides Rb_1, Rg_1 and Ro. Oral administration of RGP significantly prevented the ischemia-induced decrease in response latency, as determined by the passive avoidance test, and rescued a significant number of ischemic hippocampal CA1 pyramidal neurons in a dose-dependent manner. Intraperitoneal injections of CGS exhibited a similar neuroprotective effect. CGNS had a significant but less potent protective effect against impaired passive avoidance task and degeneration of hippocampal CA1 neurons. Ginsenoside Rb_1 significantly prolonged the response latency of ischemic gerbils and rescued a significant number of ischemic CA1 pyramidal neurons, whereas ginisenosides Rg_1 and Ro were ineffective. Postischemic treatment with RGP, CGS or ginsenoside Rb_1 was ineffective. The neuroprotective activities of RGP, CGS and ginsenoside Rb_1 were confirmed by electron microscopy counts of synapses in individual strata of the CA1 field of ischemic gerbils pretreated with the drugs. These findings suggest that RGP and CGS are effective in the prevention of delayed neuronal death, and that ginsenoside Rb_1 is one of the neuroprotective molecules within ginseng root.

Key words Passive avoidance task · Delayed neuronal death · Synapse · Ginseng root · Hippocampus

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Introduction

Ginseng root (Panax ginseng C. A. Meyer) has served as an important component of Chinese prescriptions *(Kampou)* for thousands of years. Since the introduction of ginseng root into oriental medicine, this crude drug has been thought to prevent a number of degenerative processes associated with aging, but experimental proof in support of this speculation is limited.

Petkov and Mosharrof [25] found that ginseng root improves learning ability and physical capability in aged animals. Lasarova et al. [21] also demonstrated that crude ginseng saponin significantly prevents memory impairment in mice given electroconvulsive shocks. In studies in vitro, ginseng root has been shown to facilitate survival and/or neurite extension of cultured neurons [13, 31] and to prevent the distortion of neurites by cytochalasin-B [31]. It appears that certain components of ginseng root may subserve neurotrophic functions in various pathological conditions.

Ginseng root consists of two major ingredients: crude ginseng saponin and crude ginseng non-saponin fractions. To date, more than 20 saponins have been isolated from ginseng root and identified chemically. They can be classified into three major groups according to their chemical structures: protopanaxadiol, protopanaxatriol and oleanolic acid saponins. Ginsenoside Rb_1, ginsenoside Rg_1 and ginsenoside Ro are representative substances, respectively [29]. At present, however, the question of whether or not ginseng saponins can be used for the prevention of ischemic neuronal death remains to be answered.

Mongolian gerbils with transient forebrain ischemia have been used for the evaluation of the neuroprotective actions of several drugs [3, 14, 15]. In the ischemic gerbils treated with neuroprotective agents, the response latency as determined by a step-down passive avoidance task has been shown to reflect the number of hippocampal CA1 neurons [4, 28, 33, 34]. Accordingly, we designed the present study to determine whether or not ginseng root prevents ischemia-induced learning disability and neu-

ronal loss, using the step-down passive avoidance task, and subsequent neuron counts and electron microscopic observations within the CA1 field of the gerbil hippocampus. To identify the neuroprotective ingredient(s) of ginseng root, we administered the following substances: red ginseng powder (RGP), crude ginseng saponin (CGS), crude ginseng non-saponin (CGNS), ginsenoside Rb_1, ginsenoside Rg_1 and ginsenoside Ro.

Materials and methods

Animals

Male Mongolian gerbils weighing 70–80 g (approximately 12 weeks of age) were housed communally at a constant temperature (22 ± 1°C) with a 12:12 h light-dark cycle, and given food and water ad libitum. They were handled once a week for cage cleaning. The following experiments were conducted in accordance with the Guide for Animal Experimentation at Ehime University School of Medicine.

Occlusion of the common carotid arteries

The gerbils were anesthetized with 1.5% halothane in a mixture of nitrous oxide and oxygen (1:0.75), and fixed to a stereotaxic apparatus (Narishige, Tokyo). A thermocouple needle-probe of 0.4-mm diameter (TN-800, Unique Medical Corporation, Japan) and a thermocouple meter (TME-300, Unique Medical Corporation), were used to monitor brain temperature. The thermocouple probe was inserted into a brain area located 2 mm anterior and 2 mm lateral to bregma and 2 mm ventral to the cortical surface. Then, the animal was turned over and held in a supine position. Another thermocouple needle-probe with a blunt end and a thermocouple meter assembly (2455, Yokogawa Electric Corporation Limited, Japan) were used to monitor rectal temperature [1]. Body and brain temperatures were maintained at 37 ± 0.2°C during forebrain ischemia to apply the same intensity of ischemic insult to all animals [24]. Subsequently, the concentration of halothane inhalation was lowered to 1%. Both common carotid arteries were exposed through a ventral midline incision and separated carefully from the adjacent veins and nerves. Inhalation anesthesia was terminated and immediately the common carotid arteries were clamped for 5 min with aneurysm clips. Following ischemia, the clips were removed to restore the blood flow. The vessels were inspected for the absence of blood clots and for the recovery of blood flow under an operative microscope. Thermocouple probes were then gently pulled out, and all surgical incisions were carefully sutured. Sham-operated animals were treated in the same manner except that the common carotid arteries were not clamped.

Passive avoidance task

The gerbils were trained in a conventional step-down passive avoidance apparatus which was divided into a safe platform and a grid floor [3]. The experimental chamber (22.5 × 20.0 × 19.5 cm) was made of plexiglas plate with a floor constructed of a stainless-steel grid. A scrambled DC constant current-shock generator (Muromachi Kikai Co., Japan) delivered a 0.4-mA scrambled shock through the grid. The safe platform (20.0 × 9.5 × 3.0 cm) was also made of grey plexiglas plate and fixed to one side of the chamber. Training of passive avoidance was carried out 2 days after forebrain ischemia [3]. Each animal was initially placed on the safe platform. When the gerbil stepped down onto the grid floor, it received a foot shock. Although the gerbil went repeatedly up and down between the platform and the grid, it eventually remained on the platform. This training session lasted for 300 s. After 24 h, the gerbil was placed again on the safe platform while the shock generator was turned off, and the response latency, i.e., the time until it stepped down to the grid floor, was measured. This test session also lasted for 300 s [3, 4, 26, 28, 33, 34].

Histopathological study of hippocampal CA1 region

Four days after the passive avoidance task, the animals were anesthetized with pentobarbital and perfused transcardially with heparinized phosphate-buffered saline (0.1 *M*, pH 7.4) and then with 4% paraformaldehyde-2% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.4). A brain region including the dorsal hippocampus from 0.5 to 1.5 mm posterior to bregma [32] was removed and kept in the same fixative overnight at 4°C. Four serial coronal sections, 50 μm thick, at the level 1.0–1.2 mm posterior to bregma were cut with a microslicer (Dosaka, EM, Co., Japan) for electron microscopy. The remaining dorsal hippocampus was embedded in paraffin, and 5 μm serial frontal sections were cut and stained with 0.1% cresyl violet. All neurons with intact morphological appearance in a 1-mm linear length of the hippocampal CA1 field on six serial sections, which were obtained from the level 1.20–1.23 mm posterior to bregma, were counted with an image analyzer (Nexus Co., Japan). For electron microscopy, the specimens were post-fixed with 1% osmium tetroxide for 30 min, dehydrated with a graded series of ethanol, transferred to propylene oxide and embedded in epoxy resin. The strata moleculare, lacunosum/radiatum and oriens of the CA1 field [27] were identified on semithin sections stained with 1% toluidine blue, and ultrathin sections, 70-nm thick, were cut with a Reichert-Nissei ultramicrotome (Optische Werke, Austria) and mounted on single-slot (2 × 0.5 mm) grids, which were coated with Formbar film. They were subjected to dual staining with uranyl acetate and lead citrate, and examined in a transmission electron microscope (H-12A, Hitachi, Japan). Electron micrographs of the central area (15 μm × 18.75 μm = 280 μm^2) of each stratum were taken, and intact synapses with thick apposed membranes and synaptic vesicles in the area were counted.

Drug administration

All ginseng components were extracted from Korean red ginseng (kindly supplied by Korea Ginseng and Tobacco Institute). The purity of each ginsenoside used in this study was more than 98% as determined by thin-layered chromatography and nuclear magnetic resonance [16]. RGP was suspended in distilled water and administered orally in a volume of 0.1 ml per 10 g body weight (0.6, 0.9 or 1.5 g/kg); control animals were given the same volume of distilled water (n = 6–10 for each group). CGS (50 or 100 mg/kg) and CGNS (50 or 100 mg/kg) were dissolved in isotonic saline and injected intraperitoneally; control animals were injected with the same volume of saline (n = 6–10 for each group). Ginsenoside Rb_1, Rg_1 or Ro (10 or 20 mg/kg for each) was also dissolved in isotonic saline and injected intraperiotoneally (n = 6–8 for each group) (Fig. 1). The doses of the above drugs were chosen, based on the studies of Matsuda et al. [23] and Yoshimura et al. [35].

To investigate the prophylactic effect of ginseng root on ischemic neuronal damage, each drug was administered once a day for 7 days before 5-min forebrain ischemia, and ischemia was induced 24 h after the last administration of drugs. To study the curative effects of ginseng root on ischemic neuronal loss, three drugs (RGP, CGS, Rb_1) which proved to have prophylactic actions on ischemic neuronal damage were administered once a day for 2 days after ischemia; control animals were given distilled water or saline (n = 6–8 for each group).

In sham-operated animals, distilled water was orally administered and saline was intraperitoneally administered once a day for 7 days before sham operation or for 2 days after sham operation. All experiments were done blind with respect to experimental group.

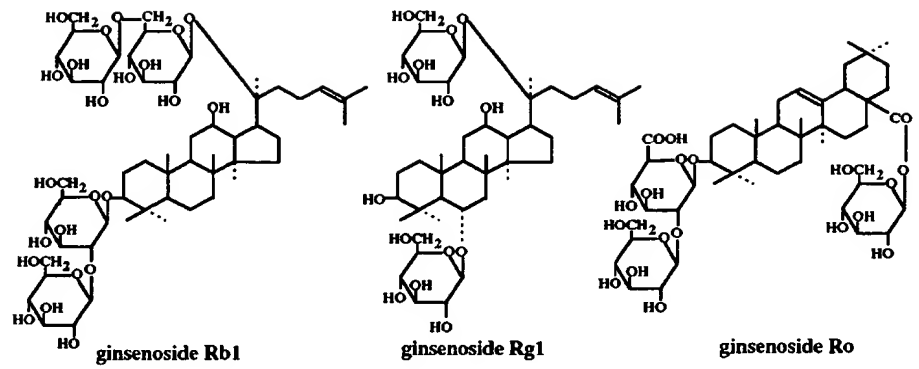

Fig. 1 Chemical structures of ginsenosides Rb_1, Rg_1 and Ro

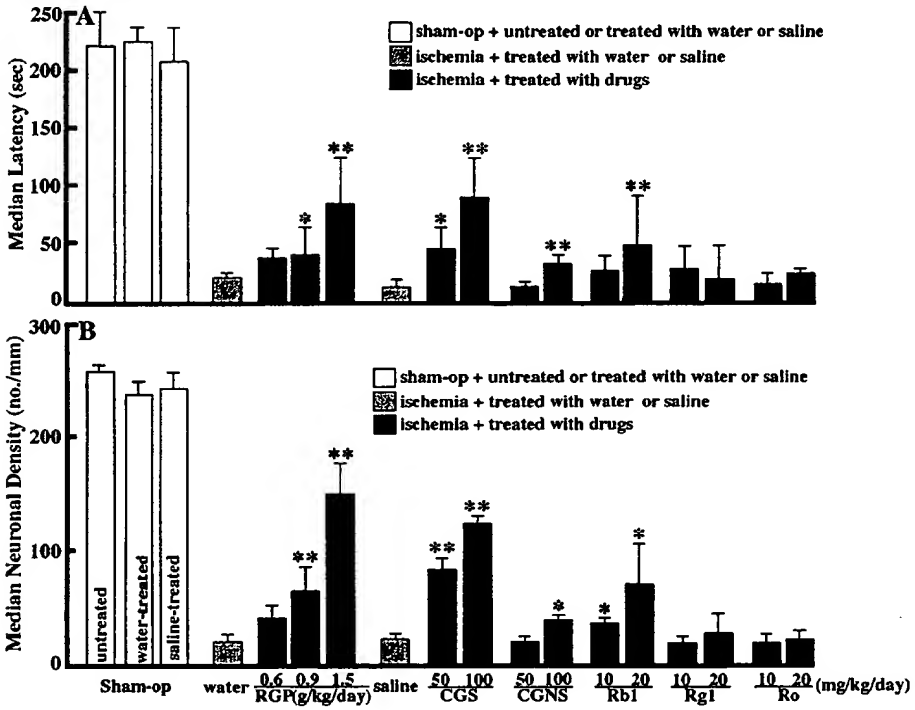

Fig. 2 Effects of red ginseng powder (RGP), crude ginseng saponin (CGS), crude ginseng non-saponin (CGNS), ginsenoside Rb_1, ginsenoside Rg_1 and ginsenoside Ro on response latency in a passive avoidance task (**A**) and on number of hippocampal CA1 neurons (**B**) in 5-min ischemic gerbils *(closed columns)*. When RGP, CGS or ginsenoside Rb_1 was administered for 7 days before ischemia, it caused a dose-dependent increase in latency in the passive avoidance task (**A**) and in the number of viable neurons within the hippocampal CA1 region (**B**), in comparison with the response latency and CA1 neuronal density of ischemic gerbils treated with distilled water or saline *(shaded columns)*. Note that CGNS pretreatment was less effective and that ginsenoside Rg_1 or Ro pretreatment was ineffective. The *open columns* indicate the median response latency and neuronal density of sham-operated *(sham-op)* animals. Each value represents median ± interquartile range (n = 6–10). $^*P < 0.05$, $^{**}P < 0.01$; significantly different from the corresponding distilled water-treated or saline-treated ischemic group

Statistics

The effects of the drugs were evaluated by the two-tailed Mann-Whitney U-test which enabled us to compare the drug-treated group with the vehicle-treated control group. Data were represented as median ± interquartile range.

Results

The median response latency and CA1 neuronal density in sham-operated gerbils were apparently higher than those in 5-min ischemic gerbils without drug treatment (sham operation without treatment: 221.0 ± 37.5 s, 254.0 ± 4.0 cells/mm; 5-min ischemia without treatment: 17.0 ± 10.0 s, 23.5 ± 7.0 cells/mm; sham operation with distilled water administration: 227.5 ± 15.5 s, 235.5 ± 11.0 cells/mm; 5-min ischemia with distilled water administration; 22 ±

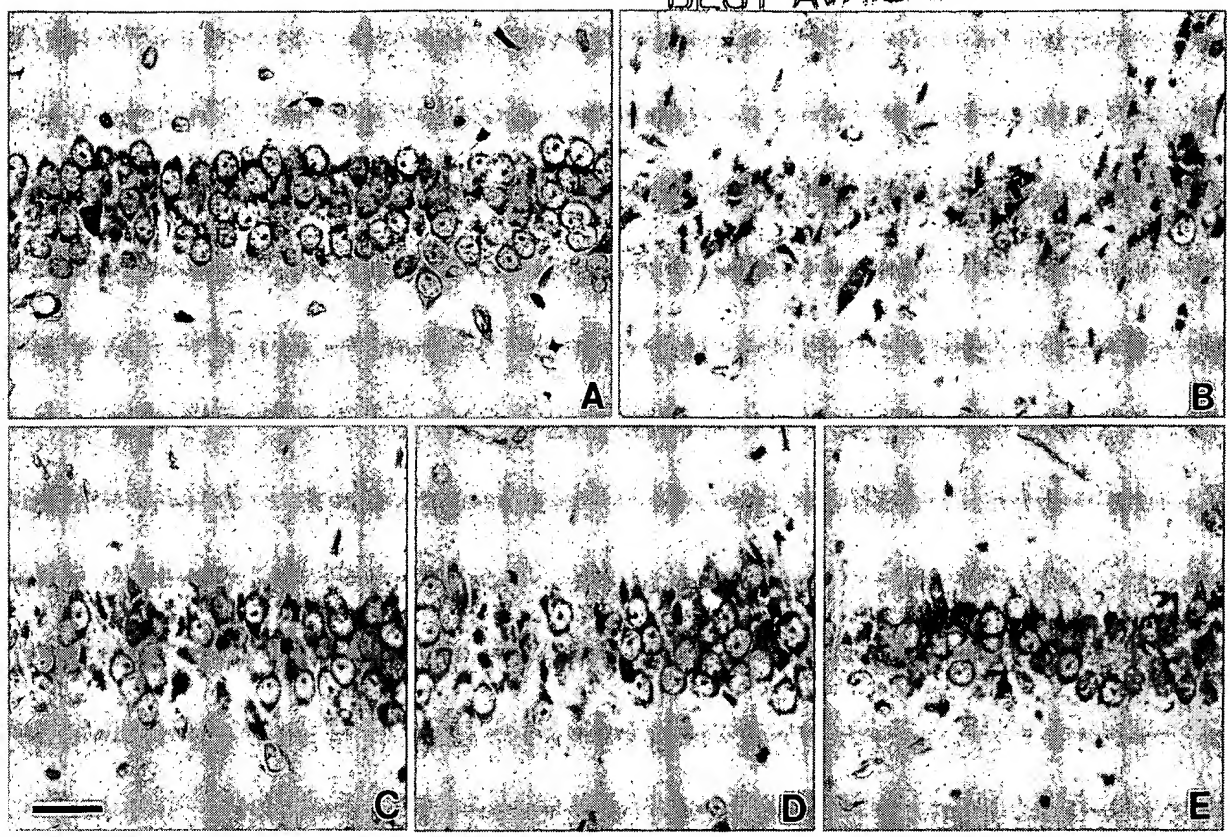

Fig. 3 A–E Photomicrographs of the hippocampal CA1 field of sham-operated and 5-min ischemic gerbils. **A** Sham-operated animals pretreated with distilled water; **B** ischemic animal pretreated with distilled water; **C** ischemic animal pretreated with RGP (1.5 g/kg per day); **D** ischemic animal pretreated with CGS (100 mg/kg per day); **E** ischemic animal pretreated with ginsenoside Rb_1 (20 mg/kg per day). Note that RGP, CGS or ginsenoside Rb_1 pretreatment rescues a significant number of hippocampal CA1 pyramidal cells. Sections are stained with 0.1% cresyl violet. *Bar* = 100 μm

4.0 s, 22 ± 5.5 cells/mm; sham operation with saline injection; 204.5 ± 27.5 s, 240.0 ± 12.0 cells/mm; 5-min ischemia with saline injection; 14 ± 15.5 s, 24 ± 4.5 cells/mm) (Fig. 2). The oral administration of RGP prior to ischemic insult significantly prolonged response latency time in a dose-dependent manner, in comparison with the corresponding vehicle-treated ischemic group (0.9 g/kg per day: $U = 6$, $P < 0.05$; 1.5 g/kg per day: $U = 3$, $P < 0.01$) (Fig. 2 A). Parallel to these findings, pretreatment with RGP caused a significant dose-dependent increase in the number of viable neurons within the hippocampal CA1 region (0.9 g/kg per day: $U = 3$, $P < 0.01$; 1.5 g/kg per day: $U = 0$, $P < 0.01$) (Fig. 2 B). Under light microscopy, viable neurons in the CA1 region of the RGP-treated hippocampus, even though less numerous than CA1 neurons in the sham-operated hippocampus, markedly outnumbered those in the counterpart of the vehicle-treated ischemic hippocampus where many pyramidal neurons were replaced by astrocytes after ischemia (Fig. 3 A–C).

Since RGP consists of two fractions, CGS and CGNS, the effect of CGS was compared with that of CGNS (Fig. 2). Intraperitoneal injection of CGS prior to ischemic insult significantly prevented the occurrence of learning disability in ischemic gerbils (50 mg/kg per day. $U = 8$, $P < 0.05$; 100 mg/kg per day: $U = 2$, $P < 0.01$) (Fig. 2 A). Subsequent light microscopic examinations demonstrated that CGS caused a significant increase in the number of CA1 pyramidal neurons (50 mg/kg per day: $U = 2$, $P < 0.01$; 100 mg/kg per day: $U = 0$, $P < 0.01$) (Figs. 2 B, 3 D). Interestingly, the mean number of CA1 pyramidal neurons after pretreatment with 100 mg/kg per day of CGS was close to that in ischemic gerbils pretreated with 1.5 g/kg per day of RGP. On the other hand, CGNS at a dose of 100 mg/kg per day had a significant but less-potent protective effect against ischemia-induced learning disorder ($U = 8$, $P < 0.01$) and degeneration of hippocampal CA1 neurons ($U = 10.5$, $P < 0.05$). The lower dose of CGNS had no significant influence on either learning disability or neuronal loss after ischemic insult.

Among the three major components of CGS, only ginsenoside Rb_1 significantly reduced the manifestation of learning disability in 5-min ischemic gerbils (20 mg/kg per day: $U = 2.5$, $P < 0.01$) (Fig. 2 A). Subsequent light microscopic examinations indicated that ginsenoside Rb_1 rescued a significant number of CA1 neurons in a dose-dependent manner (10 mg/kg per day: $U = 14$, $P < 0.05$; 20 mg/kg per day: $U = 11.5$, $P < 0.05$) (Figs. 2 B, 3 E).

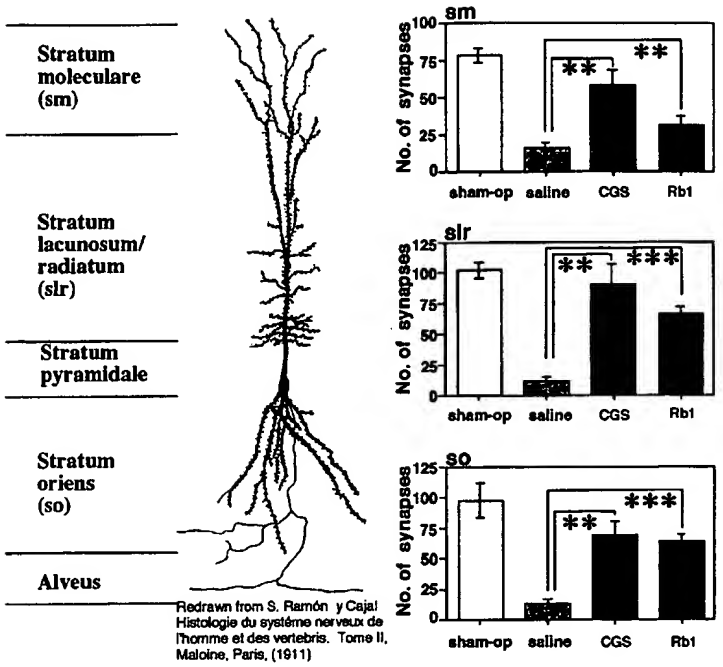

Fig. 4 Effects of CGS (100 mg/kg per day) and ginsenoside Rb_1 (20 mg/kg per day) on number of synapses within the strata lacunosum moleculare *(sm)*, radiatum *(slr)* and oriens *(so)* of the CA1 field of sham-operated and ischemic hippocampus. Note a significant increase in synapses in the CGS- or ginsenoside Rb_1-treated animals *(closed columns)*, in comparison with those in the saline-treated animals *(dotted columns)*. Since there are no differences in synapse number between CGS- and RGP-treated animals, the results of the latter animals are not shown in the figure. *Open columns* indicate the synapse number of sham-operated *(sham-op)* animals treated with saline. Each value represents median ± interquartile range ($n = 6$–10). $^{**}P < 0.01$; $^{***}P < 0.001$; significantly different from the corresponding saline-administered ischemic group

Ginsenosides Rg_1 and Ro failed to prevent the occurrence of learning disability and neuronal loss following 5-min ischemic insult (Fig. 2). Postischemic treatment with RGP, CGS or ginsenoside Rb_1 was ineffective (data not shown).

In line with the result of passive avoidance tests and neuron counts, synapses in the strata moleculare, lacunosum/radiatum and oriens of field CA1, as revealed by electron microscopy, were more numerous in ischemic gerbils treated with RGP, CGS or ginsenoside Rb_1 than in those treated with the corresponding vehicles (Figs. 4, 5). Ginseng pretreatment rescued approximately 60% of total synapses in the CA1 field, on the basis of the synapse number of ginseng-treated and sham-operated animals (Figs. 4, 5). In the vehicle-treated ischemic animals, not only postsynaptic elements of CA1 neuron origin but also presynaptic terminals arising from other neurons were frequently in the process of degeneration, whereas degenerating presynaptic terminals were rare and intact postsynaptic elements were more numerous within field CA1 of the ischemic hippocampus treated with RGP, CGS or ginsenoside Rb_1.

Discussion

The mechanism(s) by which ginseng root prevents ischemia-induced neuronal loss in the gerbil hippocampus is difficult to explain at the present moment. In cultured myocytes with ischemia-reperfusion injuries, ginsenoside Rb_1 antagonizes lipid peroxidation, scavenges oxygen free radicals and increases catalase activity [8]. Several lines of clinical and experimental evidence suggest that free radicals and lipid peroxides which are overproduced after brain ischemia are responsible for brain edema and neuronal damage [7, 9, 19, 20, 30]. Lipid peroxides in the hippocampal CA1 region are increased 24 h after cerebral ischemia in the rat [2, 6]. Hara et al. [12] reported that postischemic administration of α-tocopherol, a free radical scavenger, prevents the delayed death of ischemic hippocampal CA1 pyramidal neurons. Thus, inhibition of free radical generation or lipid peroxidation is likely to prevent the occurrence of brain edema and neuronal damage. In this context, we may account for the neuroprotective effect of ginsenoside Rb_1 by assuming that it inhibits free radical neurotoxicity and/or lipid peroxidation in ischemic brains. This speculation is also supported, in part, by the finding that ginsenoside Rb_1 has a main chemical structure similar to that of 21-aminosteroids or lazaroids which have been shown to inhibit lipid peroxidation [5, 11, 22, 37]. Since ginsenoside Rb_1 with neuroprotective activity for ischemic brain has a larger number of sugar moieties than ginsenoside Rg_1 or Ro that are devoid of neuroprotective activity, the sugar moiety that is degraded by hydroxy-radicals may also be involved in the radical-scavenging action of ginsenoside Rb_1 in cases of brain ischemia.

During 5-min forebrain ischemia, brain temperature has been shown to fall variably in individual gerbils, so the number of viable CA1 neurons after ischemia is different [1, 24]. To eliminate the effect of unstable brain temperature on ischemic neuronal loss, we kept the brain temperature at 37.0 ± 0.2°C while the common carotid arteries were clamped [24]. This enabled us to apply the same intensity of ischemic insult to all animals and to evaluate more accurately the neuroprotective effect of ginseng root in ischemic gerbils. It is of interest that even normothermic

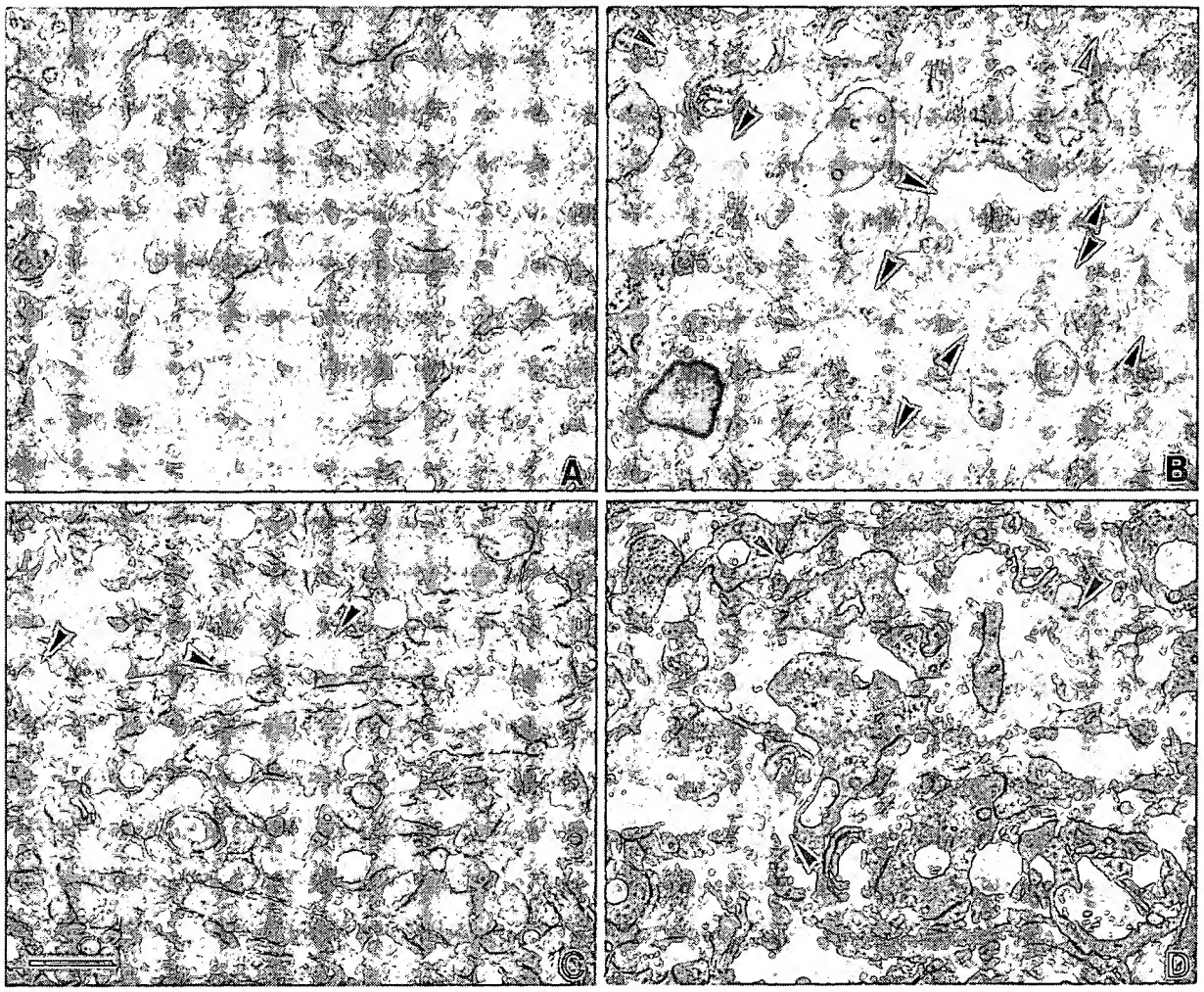

Fig. 5 A–D Electron micrographs of CA1 field of sham-operated and ischemic hippocampus. **A** Sham-operated animal treated with saline; **B** ischemic animal treated with saline; **C** ischemic animal treated with CGS (100 mg/kg per day); **D** ischemic animal treated with Rb_1 (20 mg/kg per day). Note that degenerating synaptic structures are extremely electron dense *(arrowheads)* and more numerous in **B** than in **C** or **D**. *Bar* = 1 μm

brains, which are more vulnerable to ischemia than hypothermic brains, were protected significantly by RGP pretreatment in the present study. However, ginseng pretreatment could rescue nearly half CA1 neurons from lethal ischemia; the other half degenerated after 5-min ischemia despite ginseng pretreatment. It would be of interest to see whether or not ginseng pretreatment rescues more CA1 neurons in normothermic gerbils with 3.5-min ischemia, which exhibit neuronal damage similar to hypothermic gerbils with 5-min ischemia [1, 17].

In the present study, ginsenoside Rb_1 was identified as a neuroprotective ingredient in ginseng root by the passive avoidance task and histological analysis; ginsenoside Rb_1 prevented significantly the development of learning disability and neuronal death after ischemia, whereas ginsenosides Rg_1 and Ro were ineffective. Presumably, the neuroprotective effect of CGS can be ascribed, at least in part, to the action of Rb_1. However, CGS has been shown to contain a variety of saponins with different pharmacological properties [10, 35, 36], and CGNS, even though it is less effective than CGS in affording protection for ischemic neurons, has a significant neurotrophic component(s). It will be of value to search for neurotrophic ingredients other than ginsenoside Rb_1 in ginseng root.

Kirino et al. [18] reported that presynaptic terminals in field CA1 of the hypothermic ischemic gerbil remain intact for at least 1 month after 5-min ischemia despite the appearance of marked gliosis in the field as a result of delayed neuronal death. This indicates that the presynaptic terminals in contact with the corresponding postsynaptic elements of CA1 neuron origin do not undergo retrograde degeneration in response to the loss of CA1 pyramidal neurons. In contrast, the present study showed frequent degeneration of both pre- and postsynaptic structures in

the CA1 field of the normothermic gerbil with 5-min forebrain ischemia, suggesting that retrograde degeneration of axons depends on the severity of ischemic damage of the target neurons. In ginseng-treated normothermic gerbils, degenerating presynaptic terminals were rare. This indicates that ginseng pretreatment, even though failing to rescue the postsynaptic elements of CA1 neuron origin, can preclude the secondary retrograde degeneration of the corresponding presynaptic nerve endings. The significant increase in intact synapses within field CA1 of normothermic ischemic gerbils which are pretreated with RGP, CGS or ginsenoside Rb_1 may provide a morphological basis for the functional recovery of ischemic neurons in response to treatment with ginseng root.

The inhibition by ginsenoside Rb_1 of secondary neuronal degeneration may also validate the daily use of ginseng root for the treatment of patients with chronic cerebrovascular diseases. Indeed, in a cooperative clinical study organized by the National Cardiovascular Center of Japan, the long-term (8 weeks) oral administration of red ginseng powder in patients suffering from chronic cerebrovascular diseases has been shown to ameliorate sensory impairments associated with the diseases (T. Yamaguchi and T. Omae, personal communication).

In conclusion, the present study provides experimental evidence for the ability of ginseng root to prevent ischemia-induced neuronal loss and learning disability. It is tempting to speculate that ginseng root can be used for the prophylactic treatment of cerebral infarction and/or cerebrovascular dementia.

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